T-distributed stochastic neighbor embedding (t-SNE) analysis of tumour infiltrating lymphocytes after treatment with a T cell activating therapy identifies a unique population of recruited CD8$^+$ T cells and novel options for combination immunotherapy

ABSTRACT# 4989

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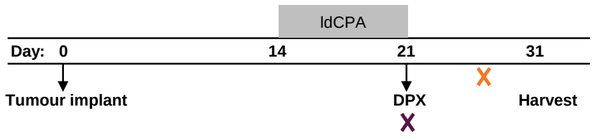
Abstract

Immune therapies for cancer seek to alter the immune profile of the tumour microenvironment (TME). A suppressive TME can counteract active immune responses by CD8$^+$ T cells by multiple mechanisms, including induction of checkpoint inhibitor (CPI) expression. DPX™ is a lipid nanoparticle-based platform that can be formulated with cancer targets to induce potent and sustained antigen-specific T cell responses. In preclinical and clinical testing previously published, combination of DPX with low dose intermittent cyclophosphamide (ldCPA) resulted in increased tumour infiltration of antigen-specific CD8$^+$ T cells. These T cells express PD-1, and combination treatment with PD-1 blocking antibody was shown previously to result in better tumour control in preclinical tumour models. In this study, we used tSNE analysis to understand changes to the immune profile induced by DPX/ldCPA treatment in the C3 model. We prioritized the expression of CPIs on tumour infiltrating CD8$^+$ T cells to identify potential combinations with blocking monoclonal antibodies to enhance tumour CD8$^+$ T cell activity. Mice (C57BL/6) bearing HPV16 E7-transformed C3 tumour cells were treated with ldCPA (20 mg/kg/day PO, 7 days), followed by subcutaneous injection with HPV16 $E7_{49-57}$ peptide in a DPX formulation (DPX-R9F). Tumours were collected for analysis 10 days after DPX-R9F treatment and a single cell suspension prepared by digestion. Immune profiling was performed by 12-color flow cytometry, to observe differences in infiltrating immune cells and CPIs. tSNE analysis of flow cytometry data was performed to analyze differences in cellular infiltrates and phenotype with treatment. We found CD8$^+$ T cells that infiltrated tumours in mice treated with DPX-R9F/ldCPA expressed most CPIs tested (including PD-1, Tim-3, Lag-3, ICOS), and fewer expressed CTLA-4. Treatment induced infiltration of a unique population of CD8$^+$ T cells that was PD-1$^+$Tim-3$^+$CTLA-4$^-$, whereas CD8$^+$ T cells present in untreated tumours were primarily PD-1$^+$Tim-3$^+$CTLA-4$^+$. Tumour challenge was performed to evaluate combination of DPX-R9F/ldCPA treatment with anti-CTLA-4 (Clone 9D9) to potentially impact these resident CD8$^+$ T cells. Mice treated with the triple combination had better control of C3 tumour growth compared to mice treated with DPX-R9F/ldCPA or anti-CTLA-4 separately. With tSNE analysis we were able to have a better model to observe differences in the CD8$^+$ population with DPX/ldCPA treatment and determine the mechanism for how certain checkpoint targets are impacting tumour infiltrating cells to further sculpt the anti-tumour response to increase efficacy of treatment.

Methods



- Day 0, C57BL6 mice were implanted with HPV16 E7-expressing C3 tumour cells (SC)
- DPX-R9F – contains peptide epitope HPV16 $E7_{49-57}$
- Low dose intermittent cyclophosphamide (ldCPA; 20 mg/kg/day PO)
- Antibody treatment as indicated in figure legends
- X → PD-1 antibody (clone RMP1-14; 200 µg/dose IP), or isotype (clone 2A3; rat IgG2a; 200 µg/dose IP)
- X → CTLA-4 antibody (clone 9D9; 100 µg/dose IP), or isotype (clone MPC-11; mouse IgG2b; 100 µg/dose IP)
- Tumours were harvested, digested, and single-cell suspensions were analysed using immunofluorescent staining
- T-distributed stochastic neighbor embedding (tSNE) analysis using FlowJo

Further information

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DPX/ldCPA induces recruitment of a unique population of CD8$^+$ T cells to tumour

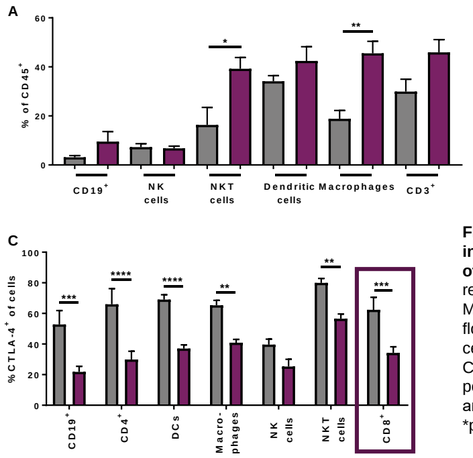


Figure 1: DPX/ldCPA treatment enhances tumour-infiltrating CD8$^+$ T cells and decreases relative abundance of CTLA-4$^+$ cells. C3 tumour-bearing C57BL6 mice (n=7-11) received treatment with DPX-R9F and ldCPA (as indicated in Methods). Tumour infiltrating lymphocytes were analysed by flow cytometry for CD19$^+$ cells, CD3$^+$ T cells, NK cells, NKT cells, dendritic cells, and macrophages (A), and within the CD3$^+$ T cells, CD4$^+$ and CD8$^+$ T cells (B). Within each cell population the relative proportion of CTLA4$^+$ cells was analysed (C). Significance calculated by student's t-test: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

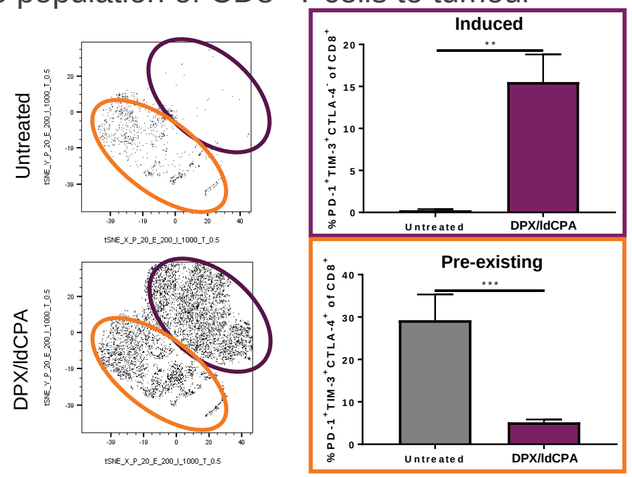


Figure 2: Tumour-infiltrating CD8$^+$ T cells in untreated C3 tumours are primarily CTLA-4$^+$, while DPX/ldCPA treatment induces additional recruitment of CTLA-4$^-$CD8$^+$ T cells. C3 tumour-bearing C57BL6 mice (n=7-11) received treatment with DPX-R9F and ldCPA (as indicated in Methods). Tumour infiltrating CD8$^+$ T cells were analysed using tSNE analysis using FlowJo (A) and distinct groups were analysed for the expression of checkpoint markers (B). Purple: PD-1$^+$Tim-3$^+$CTLA-4$^-$; Orange: PD-1$^+$Tim-3$^+$CTLA-4$^+$. Significance calculated by student's t-test: **p<0.01, ***p<0.001.

Impact of CTLA-4 blockade in combination with DPX/ldCPA treatment

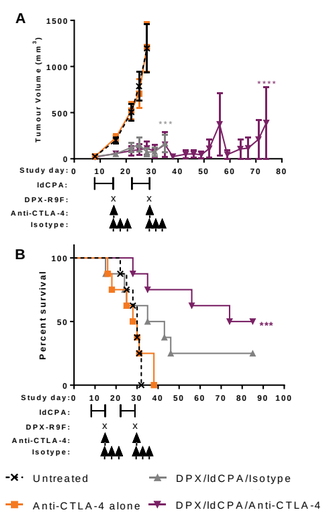


Figure 3: CTLA-4 targeting enhances therapeutic benefit of DPX based immunotherapy. On study day 0, C57BL6 mice (n=8) were implanted with HPV16 E7-expressing C3 tumour cells (SC). Groups of mice received treatment with DPX-R9F, ldCPA, and CTLA-4-targeting antibody as indicated. (A) tumour kinetics. B) survival. Significance in (A) calculated by linear regression compared to untreated: ***p<0.001, ****p,0.0001. Significance in (B) calculated by Mantel-Cox compared to untreated: ***p<0.001.

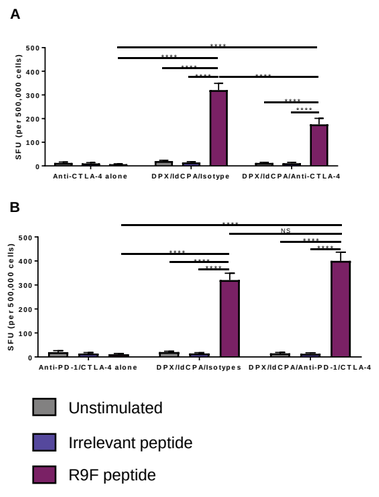


Figure 4: Blockade of CTLA-4 reduces antigen-specific immune response, which is rescued by the addition of PD-1 blockade. On study day 0, C57BL6 mice (n=5-8) were implanted with HPV16 E7-expressing C3 tumour cells (SC). Groups of mice received treatment with DPX-R9F, ldCPA (as indicated in Methods), and CTLA-4-targeting and PD-1-targeting monoclonal antibodies as indicated. Splenocytes were isolated from the mice 10 days after vaccination and assessed for R9F peptide antigen specific IFN-γ immune responses by ELISPOT assay. Significance in (A and B) calculated by Two-way ANOVA followed by Tukey's post test: NS = nonsignificant, ****p<0.0001.

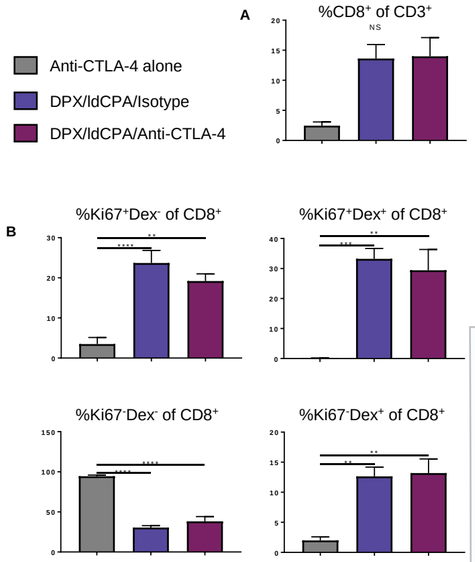
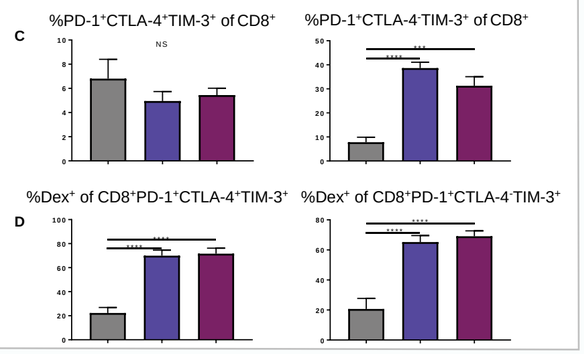


Figure 5: CTLA-4 blockade does not alter the tumour-infiltrating CD8$^+$ T cells population induced by DPX/ldCPA treatment. C3 tumour-bearing C57BL6 mice (n=6-8) received treatment with DPX-R9F, ldCPA, (as indicated in Methods) and CTLA-4-targeting monoclonal antibody as indicated. Tumour infiltrating CD8$^+$ T cells (A) were analysed to detect the proliferative capacity of antigen-specific CD8 T cells (B), as well as presence of CTLA-4 expression (C) and whether these distinct populations have different specificities (D). Significance calculated by Two-way ANOVA followed by Tukey's post-test: NS = nonsignificant, **p<0.01, ***p<0.001, ****p<0.0001.



Conclusions

- Treatment with DPX and ldCPA increases CD8$^+$ T cells, NKT cells, and macrophages in tumour microenvironment.
- Two distinct CD8$^+$ T cell populations in the C3 tumour are apparent after treatment with DPX/ldCPA.
  - Pre-existing: PD-1$^+$TIM-3$^+$CTLA-4$^+$
  - Induced: PD-1$^+$TIM-3$^+$CTLA-4$^-$
- CTLA-4 blocking antibody enhances the impact DPX/ldCPA treatment has on tumour growth and survival.
- CTLA-4 blockade may impact antigen-specific T cell response induced by DPX.
  - Therefore anti-CTLA-4 treatment may be acting on pre-existing CTLA-4$^+$ tumour infiltrating lymphocyte population.
- CTLA-4 blockade does not further alter the tumour-infiltrating CD8$^+$ population induced by DPX/ldCPA, including cellular proliferation and checkpoint inhibitor expression of peptide-specific population.

Future studies will analyse other populations and phenotypes in the C3 tumour which may be the target for CTLA-4 blockade.